TRACKING RESPONSE OF THE FRESHWATER COPEPOD *HESPERODIAPTOMUS SHOSHONE*: IMPORTANCE OF HYDRODYNAMIC FEATURES

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TRACKING RESPONSE OF THE FRESHWATER COPEPOD *HESPERODIAPTOMUS SHOSHONE*: IMPORTANCE OF HYDRODYNAMIC FEATURES

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This work is dedicated to my big sister, Summer Jackson-Healy.

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NOMENCLATURE

Before and during a tracking event: A mate tracking event is subdivided into two time periods, before and during a tracking event. Before a tracking event is the swimming behavior of males prior to detection of an emitter's trail. The onset of tracking occurs when the tracker detects the emitter's trail and performs a re-orientation in their body position within the trail. The tracker follows the trail to the emitter. In a trail mimic tracking event, the onset of a tracking event is observed when the male intersects and re-orients his body position within the trail, and follows the trail to the source of trail emission.

Encounter: A behavior where a copepod contacts another copepod or intersects a trail mimic but does not react. In a mating behavior experiment, this event is observed as an immediate escape reaction by the two copepods from one another. In a trail mimic experiment, this event is observed as a break in the visualized trail where the male passes through the trail.

Escape event: An interaction between two male copepods where the male tracker contacts another male and the immediate reaction is an escape from one another.

Capture event: An interaction between a male and female copepod where the male tracker contacts a female and subsequently captures her for copulation.

Follow: A behavior where a male intersects a previous trail produced by another copepod or a trail mimic and follows the trail. In a mating behavior experiment, the onset of a following event is seen as a male re-orients his body within the trail and follows the trail until he reaches the emitter copepod. In a trail mimic experiment, the onset of a trail-following event is seen as the male intersects the trail mimic, reacts by re-orienting his body position within the trail towards the mimic source and follows the trail towards the source.

Station holding: A behavior observed during a trail mimic interaction, where a male remains within the center line of a trail mimic by casting between the edges of a trail mimic. The result of a station holding event is a greater gross distance travelled due to casting between the trail edges and a relatively short positive net distance travelled because the male remained stationary within the trail.

Mean swimming speed (cm/s): The mean swimming speed of the tracking copepod was determined by averaging the instantaneous swimming speeds across an entire tracking event. In 3D tracking behavior, the mean swimming speed of the tracking copepod was measured both before and during the tracking event, formatted as the change in speed from before to during tracking, and compared across treatments. In 2D trail-following behavior, the mean swimming speed of the tracking male was measured only during the tracking event.

Net tracking distance (cm): The shortest distance between the location of initial trail detection to the location at the end of the tracking event.

Gross tracking distance (cm): The distance of the exact pathway taken by the tracking copepods from their initial position to their final position.

Duration of a tracking event (seconds): The total time a copepod followed another copepod or trail mimic.

Initial detection distance (cm): The net distance between the tracking male and emitting copepod at the moment the male detects the trail of the emitting copepod.

Lunge position distance (cm): The net distance between the tracker and emitter copepods at the lunge position. The lunge position was determined as a pause in motion (0.33 seconds) before the tracker copepod lunges to either touch or grab the emitter.

SUMMARY

Using three-dimensional Schlieren-based videography, males of the freshwater alpine species *Hesperodiaptomus shoshone* (Wyoming) were found to follow both conspecific females and conspecific males, remaining 0.45 ± 0.13 cm (male) and 0.56 ± 0.13 cm (female) from the lead copepod for 0.91 ± 0.35 seconds (male) and 0.84 ± 0.46 seconds (female). Trail following is initiated when the male makes a rapid reorientation. Chemical pheromones either were not produced by the female or were not detected by the male because males would follow trail mimics composed of female-conditioned water. Using unconditioned water, males were found capable not only of following trail mimics but they showed a preference, quantified as a higher follow frequency, of trails running at speeds matching that of their female mate. Remarkably, the male copepods always followed upstream, micro-casting between the edges of the trail to remain on track. Trails flowing at speeds matching their mate's swimming speed were followed for a longer period of time and at greater gross distance. As the flow speed of the trail mimic increased, the distance the copepod would advance would decrease until the threshold speed of 2.30 cm/sec at which it would not follow a trail and only station hold. Station holding has never been observed before for copepods and may represent an adaptive behavior to avoid being washed out of their resident alpine pond. At speeds greater than that evoking station holding, the stream seemed to push the copepod out of the flow even though the copepod would make repeated efforts to swim up the stream. This research revealed a behavior not documented before: instead of relying on discrete pulses of flow left by hopping copepods, this high alpine lake copepod followed smoothly swimming mates or continuously flowing thin streams, relying only on sensing hydrodynamic cues.

CHAPTER 1

TRACKING RESPONSE OF THE FRESHWATER COPEPOD *HESPERODIAPTOMUS SHOSHONE*: IMPORTANCE OF HYDRODYNAMIC FEATURES

1.1 Introduction

Sexually reproducing organisms face the primary challenge of finding a conspecific mate of the opposite sex. One heterosexual organism: planktonic copepods - can be especially challenged because they are small; ~1 mm, they live in a three-dimensional environment: the ocean, and their closest copepod neighbor can be 10-100's of body lengths away. Given the large separation between likely mates in such dilute populations, encountering a conspecific can be infrequent (Gerritsen 1980, Buskey, 1998). Copepods can increase their chances of encountering mates by actively searching and by having the capability to remotely detect the presence of potential mates (Gerritsen and Strickler, 1977; Haury and Yamazaki, 1995; Kiorboe, 2006). Chemoreception and hydromechanoreception are two primary sensory modalities that copepods use to remotely detect conspecific mates (Mauchline, 1998).

Many chemoreceptive male copepods use chemically scented trails to remotely detect and track down a potential female mate (*Temora longicornis,* Doall et al., 1998; *Calanus marshallae*, Tsuda and Miller, 1998; *Centropages typicus,* Colin, 1995; Bagoien and Kiorboe 2005a; *Centropages hamatus*, Colin, 1995; *Eurytemora affinis*, Seuront, 2013). Chemically scented trails are produced when a smoothly swimming female disturbs the surrounding fluid, and produces a continuous trail in her wake in which she releases her diffusible chemical exudates. Males detect the chemically scented trail and follow the trail to the female source for mating. Chemically scented trails increase the

probability of encountering a mate because the trail increases the active space from that which surrounds the organism to a trail which increases the size of the active space from 1 mm of the body size to 100 mm or the length of the trail. Therefore, the probability of encountering a mate increases through the detection of a trail rather than a point source of odorant. In addition to long range mate detection, chemically scented trails can inform the male tracker on the species, sex, and sexual receptivity of the female cue releaser. For example, male *Diapotmus leptopus* exhibited mate searching behavior (in terms of frequency of turns and swimming speed) when exposed to the smell of conspecific gravid females as opposed to conspecific non-gravid females and males (van Leeuwen and Maly, 1991). Male *Oithona davisae* increased their mean swimming velocity and exhibited mate-searching behavior in water conditioned with a conspecific virgin female, but not a conspecific mated female or male (Heuschele and Kiorboe, 2012). Male *Eurytemora affinis* exhibited a preference for chemically scented trail mimics that were scented with the smell of conspecific non-ovigerous females, as compared to conspecific ovigerous females and males (Seuront, 2013).

In addition to chemoreception, aquatic animals can sense hydrodynamic cues and use them to detect the presence of nearby predators, prey and conspecifics. Hydrodynamic cues are generated when an animal swims through water, disturbs the surrounding fluid, and generates a wake behind it. This wake can be specific to an animal depending on its size, swimming behavior, and speed. Other nearby animals can detect these specific hydrodynamic cues and track them to find the exact location of the cue releaser (Hanke et al., 2000; Pohlman et al., 2001; Pohlman et al., 2004). Copepods are able to distinguish between the wakes of nearby predators, prey and conspecifics and react appropriately. In opposition to chemoreception of trail-like cues, copepods that detect hydrodynamic disturbances sense animals that hop through the water column at a short detection distance of 1-2 body lengths from the source. For example, *Euchaeta rimana* detect hydrodynamic disturbances from nearby hopping prey that are up to 2

body lengths away to determine their location for attack (Doall et al., 2002). Fields and Yen (2002) exposed a female *Euchaeta rimana* to a jet that mimicked an artificial hydrodynamic disturbance from either a prey or predator. The female evoked a capture response towards the prey mimic and an escape response towards the predator mimic. It was concluded that female *Euchaeta rimana* can discriminate between hydrodynamic cues of a predator and prey and react appropriately.

Hydrodynamically mediated mate finding behavior has only been described for males following a hopping female, and not a trail-like cue from a smoothly swimming female. For example, male *Acartia tonsa* detect the presence of a nearby potential mate through small fluid mechanical disturbances generated by a hopping conspecific female. As the male hops shortly after the female, the distance between the two copepods decreases until the male lunges and grabs the female to mate (Bagoein and Kiorboe, 2005b).

There are costs in producing chemical and hydrodynamic cues, but these costs outweigh the benefit of increasing the probability of encountering a potential female mate. Hydrodynamic cues produced by hopping can be energetically expensive to create, but these hops produce large disturbances of the surrounding fluid medium that in turn cue a females' presence to nearby conspecific males (van Duren et al., 1998). For example, female *Temora longicornis* generated short hops as a reaction to chemical exudates released by conspecific males (van Duren et al., 1998). This behavior advertised the female presence to nearby males and thus increased mate encounter rates for reproduction. Similarly, chemical cues can be energetically expensive for a female to produce because of the high rate of molecular diffusion in water (Dusenberry and Snell, 1995). However, the cost of chemical production may be much lower for plankton greater than 100 micrometers in spherical radius because larger plankton can produce greater yield of chemical product to combat the fast diffusion rate of chemicals in water (Dusenberry and Snell, 1995).

The current study focused on a population of *Hesperodiaptomus shoshone* that was collected from Rock Pond in the Shoshone National Forest, Wyoming, USA. *Hesperodiaptomus*, a genus of large freshwater calanoid copepods, is commonly found in high alpine regions of Northern America (Yen et al., 2011). Their mating season is short, spanning from 1 to 3 months, and is determined by the onset of seasonal melting and freezing of their home lake. Information on their mate-finding behavior has been investigated from a population of *H. shoshone* in California, in which males preferentially followed female pheromone trail mimics scented with female diffusible chemical exudates, compared to male and non-scented trail mimics (Yen et al., 2011). I predict that *H. shoshone* (Wyoming) may rely on chemical cues to find their mates due to the findings from the California population (refer to Yen et al., 2011), but also due to their relatively large size (refer to Dusenberry and Snell, 1995). If *H. shoshone* (Wyoming) does not display a reaction to chemical cues, then it would be important to assess the role of hydromechanical cues in mate detection. In addition to chemical cues, aquatic organisms also disturb the fluid as they swim, leaving hydrodynamic cues. Likely sources of these cues are other residents of the lake community. I predict that *H. shoshone* (Wyoming) may rely on hydrodynamic cues to find a mate, and can discern the wakes of their conspecific mate from other lake resident wakes (e.g. their *Daphnia spp*. prey and fairy shrimp residents).

Not only can these cues indicate the presence of females and males, I hypothesize that males are able to detect cues in their environment that inform them of the presence of co-occurring *Daphnia spp*. prey and fairy shrimp residents. Local flow in the lake also may provide useful information about their habitat, e.g. cold melt water sinking, or exit flow from the lake. To test these hypotheses, I asked three questions: (1) Do males of *H. shoshone* rely on chemical or hydromechanical cues to find their mates? (2) If chemical cues are important, is there any specificity in this cue? (e.g. are males able to detect a difference in the diffusible chemical exudates of males and females) and (3) If

mechanical cues are important, is there any specificity in this cue? (e.g. are males able to distinguish the hydrodynamic wake of a conspecific mate from other lake residents).

1.2 Methods and Materials

1.2.1 Plankton collection and laboratory care

Copepods (*Hesperodiaptomus shoshone*) were collected from Rock Pond in the Shoshone National Forest in Wyoming USA. The copepods were collected by hand-retrieving a weighted ½ -m 333 um-mesh plankton net thrown 20 ft. from the shore in an alpine glacier-fed pond at 10,789 ft. elevation. Three annual collections over 3 successive years were done on: (1) August 16, 2011 for use in mating behavior between males and females; (2) July 30, 2012 for use in conspecific behavior between the same sex; and (3) August 5, 2013 for use in trail mimic bioassays. For the 2013 collection, two additional co-occurring zooplankton species (fairy shrimp and *Daphnia spp*.) were collected from Rock Pond for behavioral analysis of their hydrodynamic cues.

After on-site collection, the copepods were transferred into thermoses at densities of 50 copepods/1-L lake water, insulated with ice packs, and shipped overnight to the Georgia Institute of Technology in Atlanta, Georgia. Upon shipment arrival, water quality analyses determined that their natural lake water was between a pH of 5-6, and their travel temperature between 4-10˚C. Copepods were allowed to slowly reach the ambient lake temperature (12˚C) in a temperature controlled room (10-14˚C). Upon temperature acclimation, 100-150 copepods were transferred to 20-L containers prefilled with 15-L of artificial lake water (EPA medium) with the pH matched to their natural lake water. Copepods were fed daily a mixed diet of lab hatched *Artemia sp*. and cultured *Daphnia spp.*. Their water was mildly aerated and cleaned on a weekly basis.

1.2.2 Experimental design

Mating behavior. Initial observations of *H. shoshone* mating behavior revealed that males tracked conspecific males and females. The mating behavior experiment was performed to test the hypothesis that *males recognize the cues generated by conspecific males and females and results in a difference in tracking behavior.* Three treatments were used in the mating behavior experiment: two control treatments and one experimental treatment. The two control treatments were: (1) males interacting with other males; and (2) females interacting with other females. These single sex treatments were conducted to determine the baseline behavior of male and female interactions. The experimental treatment was males interacting with females to determine if male tracking behavior differed between tracking a female, as compared to a male.

Mating Behavior Experiment. Males and females were separated for at least 24 hours prior to each experimental trial. This step was repeated to ensure that males had not mated for the past 24 hours and would have an available spermatophore since it takes about 1 day to generate a single spermatophore (Ceballos & Kiorboe, 2010). At the start of each experiment, 10 copepods of the same sex (male or female) were transferred to the experimental tank, which contained 600-mL of nanopure EPA water (pH 6, 12˚C). After a 10-minute period to adjust to being transferred, the second set of copepods (male or female) was transferred to the tank and the copepods were allowed to interact for 2 to 4 hours. For each treatment trial, the order at which copepods entered the tank, either as the first or second group, was switched to prevent experimental bias.

Chemical Trail Following Behavior. If there was not a significant difference in the behavior of males tracking other males and females, then there are three likely explanations for this result: (1) males and females of *H. shoshone* release the same diffusible chemical exudates (2) males and females of *H. shoshone* do not release diffusible chemical exudates or (3) males of *H. shoshone* do not rely on diffusible chemical exudates to find a mate. To assess the importance of chemical cues in mate

detection, I tested the hypothesis that *males are sensitive to conspecific male and female diffusible chemical exudates*. Male copepods were exposed to three different scent treatments: male conditioned water, female conditioned water, and water that the copepods were maintained without copepod conditioning (Table 1). Also, these treatments allowed us to determine *if males were able to detect and follow sex specific diffusible chemical exudates from conspecific males and females*.

Copepods were sorted by sex and separated for 24 hours to begin collecting the diffusible exudates for the scent treatments (Table 1). After the separation period, the copepods were fed a mixture of lab hatched *Artemia sp.* and *Daphnia spp*. and were allowed to feed for three hours. After the feeding period, copepods were placed in nanopure EPA water for 12 hours at a constant temperature of 12˚C in order to collect their diffusible chemical exudates. Following this incubation period, the scented water was collected by straining the copepods using a 250 um-mesh size filter and retaining the remaining scented water for trail treatments. The scented water was stored in sterile 20-mL glass scintillation vials at -80˚C, until used for a trail mimic bioassay. All glassware was soaked in 10% strength hydrochloric acid for at least 24 hours prior to scent collection to remove chemical remnants from previous experiments.

Hydrodynamic Trail Following Behavior. If there was not a significant difference in the trail following behavior of *H. shoshone* males between the scented trail treatments, then it would be important to assess the role of hydromechanical cues in mate detection. In addition to chemical cues, organisms that move through water also disturb the fluid, leaving hydrodynamic cues. Likely sources of these cues are other residents of the lake community. These residents included: (1) males and females of *H. shoshone* [2.41 ± 0.02 mm, n = 20 (males) and 2.59 ± 0.02 mm, n = 20 (females)]; (2) fairy shrimp [10.65 mm, n = 1], and (3) *Daphnia spp.* [1.34 mm, n = 1]. To assess the importance of hydromechanical cues, I tested the hypothesis that *males are able to distinguish between the hydrodynamic wake of their mate versus that of other residents in their environment*.

The speed of the trail mimic was adjusted to match that of male and female copepods, *Daphnia spp.* prey, and resident fairy shrimp. Measurements were made of the swimming speeds of lake residents that co-occur with *H. shoshone* to provide a cue that matches that experienced by *H. shoshone* in their natural environment. The swimming speeds for *Daphnia spp.* and fairy shrimp were determined using the same methodology as determining the average swimming speed of three-dimensional mating behavior in *H. shoshone*. Based on these analyses, four separate experiments were designed with trail mimics (Yen et al., 2004) set to match a continuous scale of increasing flow speeds within the range of swimming speeds of the aforementioned lake residents.

Trail Mimic Bioassay. Yen et al. (2004) developed a mimic of one type of mating cue, the wake left behind a swimming copepod. This mimic can be varied in terms of its chemical content as well as its hydrodynamics. Here, the trails were scented with the odor of males or females of *H. shoshone* and water that lacked their scent (control treatment). The speed of the trail mimic was modified by varying the trail hydrodynamics. Here, the trail mimic was modified to match the mean swimming speeds of the lake residents that were collected with *H. shoshone* copepods. Dextran, a high molecular weight sugar, was added to each trail treatment to increase the difference in the refractive index of the water and the trail mimic. This enabled visualization of the trails and the male trackers. At the start of each experimental trial, 10 males were added to the experimental tank, which contained 600-mL of nanopure EPA water (pH 6, 12˚C). The trails were placed in the water after the acclimation period and males were allowed to interact with the trails for the duration of the experiment. The order at which the trails were arranged in the tank was randomized for each trail replicate to prevent experimental bias.

1.2.3 Visualization and recording behavior

To visualize the copepods and trail mimics, a Schlieren optical pathway was used which enhanced the difference in the refractive index of the copepods and trail mimics from the surrounding water medium. All observations were recorded in the dark with a near-infrared laser that was used to illuminate the copepods and trail mimics. The three-dimensional movements of mate-tracking copepods were obtained by tracking images produced in a Schlieren-based videography system. This system produced paired two-dimensional images of the same copepod from orthogonal views onto a single image (Strickler, 1998; Doall et al., 1998). This single image characterized the position of a copepod with two sets of coordinates, (X, Z_1) and (Y, Z_2). The X and Y coordinates were taken directly from the video image, and the Z coordinates, Z_1 and Z_2, were taken as a mean Z value. A 1-centimeter calibration stick was placed inside each experimental tank at the beginning of a trial. The calibration stick was used to convert the X and Y coordinate pixels into useful measurements. Two-dimensional movements of trail-tracking copepods were produced as a single two-dimensional image by the Schlieren-based videography system. The exact position of a trail-tracking copepod was characterized by X and Y coordinates, which were then converted from pixels into useful metrics. All mating and trail mimic experimental trial observations were recorded onto a DVD format and digitized for behavioral analysis.

1.2.4 Behavior tracking and kinematic analysis

The mating and trail following behaviors were quantified in terms of both total number of events per replicate and kinematic analyses of behavioral events. Copepod tracking events were included in kinematic analysis only if the two focal copepods in the tracking event were visible in three-dimensions. Acceptable three-dimensional clips were seen as copepods that were centrally located away from the walls of the experimental tank, and encompassed swimming behavior occurring before the onset of tracking, during the

tracking event, and up to the final point of contact between the two copepods (see nomenclature for a detailed description of "before and during" a tracking event). For trail-following behavior, clips of trail tracking events were included in analysis if they included male swimming behavior from the point of trail entry to the point of trail exit. Clips of mating and trail-following behavior were spliced using Solveig MM Video Splitter 3 software, converted to a readable format (.avi) for the tracking program using Prism Video Converter version 1.82. Tracking paths were analyzed using Hedricks software for MatLab (Hedrick, 2008). The Hedricks software allowed for manual tracking of individual copepod movement from each video frame by recording the X, Y, and Z coordinates (three-dimensional mating behavior) or the X and Y coordinates (two-dimensional trail-following behavior) in the images using pixel locations. These coordinates aided in producing useful kinematic measurements for behavioral analysis.

1.2.5 Kinematic measures for behavioral analysis

To discern differences in tracking behavior, several metrics were needed and used to analyze three-dimensional tracking behavior: mean swimming speed, net tracking distance, gross tracking distance, duration of the tracking event, initial detection distance, and the distance at the lunge position (see nomenclature for descriptions of each kinematic metric). Several metrics were used to analyze male behavior in two-dimensional trail mimic tracking events: mean swimming speed, net tracking distance, gross tracking distance, and the duration of a tracking event (see nomenclature for descriptions of each kinematic metric).

If the males show a difference in the mean values of these kinematic metrics between tracking a male and female copepod or a trail mimic, then it will be concluded that the cues are different. It will be concluded that the cue is more detectable to the males if either of the following occurs: (a) the males follow a cue at a greater mean swimming speed (b) the males follow a cue for a greater net or gross distance (c) the

males follow a cue for a longer duration (d) the males show a difference in the initial detection distance between tracking a male and female copepod (e) the males show a difference in the lunge position distance between tracking a male and female copepod.

1.2.6 Statistics

The total number of observations for each type of behavioral event were transformed into proportional data (the total number of observations for a behavioral event/total number of observations for that treatment) with 95% confidence limits (Vassar Stats) to examine differences between treatments. The mating behavior events included encounters, escape events, and capture events. The trail tracking behavior events included encounters, follows, and station holding events.

A multiple analysis of variance test (MANOVA; JMP Pro 11 statistical software program) was used to determine treatment effects on the mean kinematic metric values. A summary of the experiments and their replicates can be found in table 1.

Table 1: Experimental design: The following data were collected for each experiment. In the chemical trail mimic experiment, each scent treatment was collected at a ratio of 1 copepod per 10-mL of nanopure EPA water. The density of copepods used for each scent treatment can be found below the chemical trail treatment. An experimental tank was considered a replicate and, therefore all the tracking measurements within a replicate were taken as a single mean for that replicate.

Experiment	Treatments	Experiment Duration (min)	Replicates used in number of events	Replicates used in tracking behavior	Total tracks analyzed for behavioral analysis
Mating Behavior	Males only	120	3	1	17
	Females only	120	4	0	0
	Males with females	120-240	8	10	59
Chemical Trail Mimic	Control scent (0 animals/100-mL water)	60	6	4	55
	H. shoshone female scent (50 females/500-mL water)	60	6	4	62
	H. shoshone male scent (10 males/100-mL water)	60	6	4	61
Hydrodynamic Trail Mimic	Experiment 1	60	6	4	144
	Experiment 2	18	5	4	133
	Experiment 3	6	5	5	78
	Experiment 4	5	5	5	17

1.3 Results

1.3.1 Mating behavior

Quantifying the behavioral interactions. Two types of mate-tracking behavior were observed among the treatments: escape and capture events (Figure 1). In both behaviors, males would track the cue emitting copepod but, upon contact would either escape or capture the emitter. In the treatment with only females present at a density of 20 females/600 mL, encounters were rare (less than 1/hour) in contrast to more than 10/hour for the treatment with only males present at the same density. Digitized images of female

copepods were statistically larger (prosome length, 0.32cm ± 0.03cm; width, 0.15cm ± 0.02cm, n = 20) than the digitized images of the male copepod (prosome length, 0.27cm ± 0.04cm; width, 0.13 ± 0.03cm, n = 20). In other copepod studies, researchers typically cannot distinguish males from females during a tracking event. This study is at a particular advantage in that the sexes can be distinguished in each behavioral interaction. Based on size differences to distinguish males from females in the videos, it was observed that when males interacted with males, they would escape from each other. However, when males interacted with females, the male would capture the female. In comparing the proportion of the total number of encounters, escape events and capture events between treatments with 95% confidence limits, the main result was the presence of capture events only in the treatment with males and females present (Figure 2).

Kinematic metrics. The behavior of the tracker males that engaged in escape events did not differ between treatments of males only or males with females, the replicates from both treatments were pooled (MANOVA, identity response, $F_{(6, 1)} = 0.52$, Wilks' Lambda, $p = 0.66$). There was not a significant difference in the mean values of the kinematic metrics when comparing the male tracker behavior in tracking another male (escape events) and a female (capture events) (MANOVA, identity response, $F_{(6, 8)} = 1.24$, Wilks' Lambda, $p = 0.38$) (Table 2).

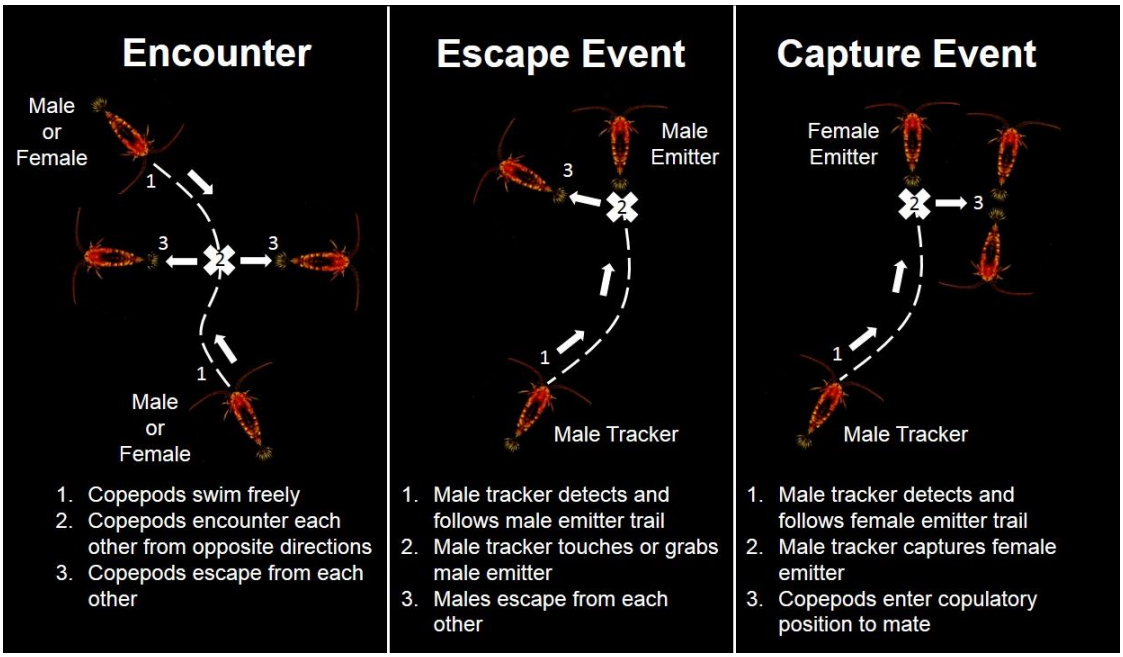

Figure 1: Two types of tracking behavior were observed between interactions of male and female copepods. A brief description for each type of interaction can be found below the image.

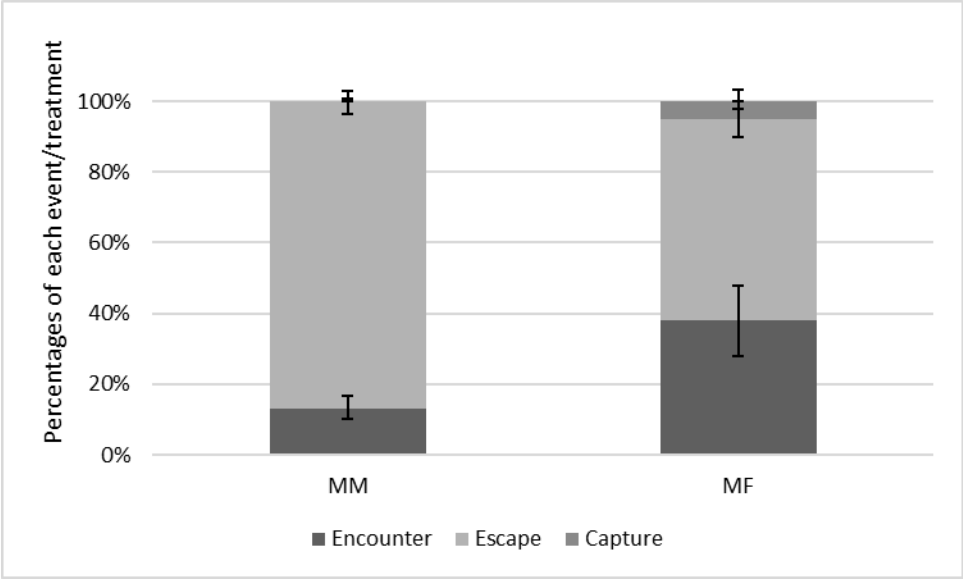

Figure 2: Proportion of copepod interactions classified as encounters, escape events, and capture events between male-male (MM) and male-female (MF) treatments with 95% confidence limits. Escape events occurred between two males regardless of the treatment. The proportion of encounters (n = 69 events), escape events (n = 403 events), and capture events (n = 0 events) in the MM treatment differed from the proportion of encounters (n = 187 events), escape events (n = 300 events), and capture events (n = 26 events) in the MF treatment. Note that capture events only occurred in male-female treatments.

Table 2: A comparison of kinematic metrics for a male tracking another male (escape events) and tracking a female (capture events). There was not a significant difference in the mean values of the kinematic metrics between escape and capture events (MANOVA, identity response, $F_{(6, 8)} = 1.24$, Wilks' Lambda, $p = 0.38$). The values of the kinematic metrics are expressed as the mean value ± standard deviation.

Kinematic Metric	Escape Events	Capture Events
Initial detection distance (cm)	0.45 ± 0.13	0.56 ± 0.13
Time spent following a trail (sec)	0.91 ± 0.35	0.86 ± 0.46
Avg. speed during tracking (cm/s)	1.07 ± 0.36	1.20 ± 0.42
Change in speed (cm/s)	0.28 ± 0.31	0.12 ± 0.12
Net distance tracked (cm)	0.60 ± 0.29	0.85 ± 0.39
Gross distance tracked (cm)	1.07 ± 0.77	1.10 ± 0.52
Lunge position distance (cm)	0.17 ± 0.07	0.27 ± 0.09

1.3.2 Chemical trails

Quantifying the chemical trail behaviors. Two types of behavior were observed, in that after intersecting the trail, males would either not follow a trail mimic ("trail encounter") or follow the trail mimic ("trail follow") (see nomenclature). In comparing the proportion of the total number of trail encounters and follows between trail treatments with 95% confidence limits, a significant difference was not found (Figure 3).

Kinematic metrics. There was not a significant difference when comparing the mean values of the kinematic metrics between trail treatments (MANOVA, identity response, $F_{(8, 12)} = 1.04$, Wilks' Lambda $p = 0.46$) (Table 3).

The experiments to evaluate sensitivity to chemicals were run at speeds (0.24 cm/s ± 0.02 cm/s) that were less than those of the female copepods (0.98 ± 0.45 cm/s). In experiments without scent, such speeds (0.07 – 0.29 cm/s) also evoked fewer responses. Many organisms have different senses, capable of detecting cues of different modalities. Sometimes, the presence of one cue can enhance the response to other cues. Since copepods are known to have chemical and hydrodynamic sensors, a next experiment on the role of multi modal sensitivity using the most detectable flow speed and varying the chemical composition could reveal a chemosensory capability.

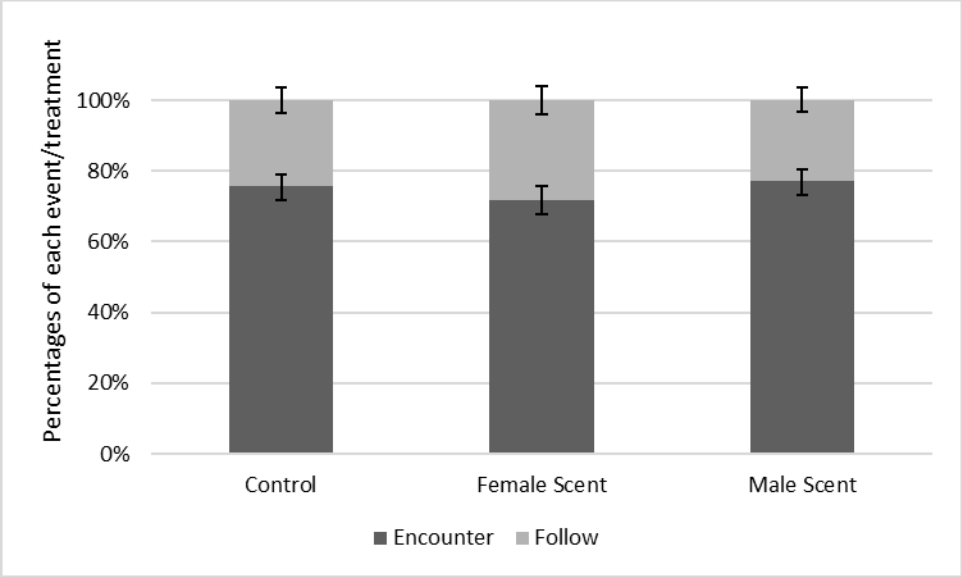

Figure 3: Proportion of copepod trail interactions classified as trail encounters and follows between trail treatments with 95% confidence limits. The treatments did not differ in the proportion of encounters (control = 405, female scent = 346, male scent = 423) and follows (control = 130, female scent = 136, male scent = 126).

Table 3: A comparison of kinematic metrics between chemical trail treatments. The kinematic metric values were not significantly different between chemical trail treatments (MANOVA, identity response, $F_{(8, 12)} = 1.04$, Wilks' Lambda, $p = 0.46$). The values of the kinematic metrics are expressed as the mean value ± standard deviation.

Kinematic Metric	Control Scent	Female Scent	Male Scent
Duration of tracking event (sec)	1.35 ± 0.22	1.04 ± 0.07	1.16 ± 0.13
Average Trail Tracking Speed (cm/s)	0.91 ± 0.12	1.05 ± 0.09	1.00 ± 0.06
Net Distance Tracked (cm)	1.27 ± 0.12	1.14 ± 0.17	1.29 ± 0.10
Gross Distance Tracked (cm)	1.51 ± 0.12	1.29 ± 0.17	1.47 ± 0.11

1.3.3 Hydrodynamic trail experiments

Quantifying the hydrodynamic trail behaviors. Four experiments were designed with trail mimics set to match a continuous scale of increasing flow speeds that comprised swimming speeds of *Daphnia spp.* [0.48 ± 0.10 cm/s, n = 20 trajectories], fairy shrimp [1.35 ± 0.47 cm/s, n = 20 trajectories], and conspecific copepods [*H. shoshone* males (0.97 ± 0.43 cm/s, n = 20 trajectories) and females (0.98 ± 0.45 cm/s, n = 20

trajectories)]. Two types of trail behavior were observed: following and station holding events (see nomenclature). Due to the design of the hydrodynamic trail mimic experiments, a statistical analysis to compare values between trail experiments could not be performed. However, the total number of events was normalized across experiments using a metric of frequency, which was expressed as the mean number of following and station holding events per minute. A general trend suggested males increased their frequency of activity as the trail speed increased, with a peak between 0.53-0.60 cm/s (Figure 4c). Furthermore, males exhibited a change in behavior at the (0.53 cm/s) trail speed treatment to include station holding behavior. As the trail speed increased, males decreased in the mean number of following events, (Figure 4a) and increased in station holding events (Figure 4b).

The following results are reported for each hydrodynamic trail experiment. In the first experiment (Figure 5), males followed faster trail speeds (0.26 cm/s and 0.29 cm/s) proportionally more than slower trail speeds (0.07 cm/s and 0.10 cm/s). In the second experiment (Figure 5), males followed the fastest trail speed (0.60 cm/s) proportionally more than the two slower trail speeds (0.29 cm/s and 0.44 cm/s). Males exhibited station holding behavior only on the fastest trail speed treatment (0.60 cm/s). In the third experiment (Figure 5), males followed the slowest trail speed (0.53 cm/s) proportionally more than the two faster trail speeds (0.64 cm/s and 1.23 cm/s). Males exhibited station holding behavior on all trail speed treatments, but this behavior was proportionally greater on the fastest trail speed (1.23 cm/s). In the fourth experiment (Figure 5), there were no observable trends or significant differences between the proportion of trail encounters, follows, or station holding events between the trail speed treatments.

Kinematic metrics. Due to the constraints of the experimental design, I cannot report statistical significance between trail experiments. However, the results strongly suggest that the male copepod showed a speed-related modification in its response. Males followed a copepod and daphnia (0.53 cm/s) trail speed mimic at a greater speed and net

distance tracked compared to treatments that mimicked only a copepod (0.64 cm/s) or an overlap in speeds by copepods and fairy shrimp (1.23 cm/s) (Table 4). Lower speed trails were followed for a shorter period of time, tracked at a faster speed, for a greater net distance and shorter gross distance (Tables 6, 7, and 8).

The following results are reported for each hydrodynamic trail experiment. In the first experiment (Table 4), there was not a significant difference in the mean values of the kinematic metrics between the trail speed treatments (MANOVA, identity response, $F_{(12, 13.52)} = 1.46$, Wilks' Lambda, $p = 0.25$). In the second experiment (Table 4), there was a significant difference in the mean values of kinematic metrics between trail speed treatments (MANOVA, identity response, $F_{(8, 12)} = 3.33$, Wilks' Lambda, $p = 0.03$). The speed at which males followed a trail was slower for the (0.60 cm/s) trail speed treatment compared to trail speeds of (0.29 cm/s) and (0.44 cm/s) (one-way ANOVA, F ratio = 17.36, df = 2, $p < 0.001$). In the third experiment (Table 4), there was a significant difference in the mean values of the kinematic metrics between the trail speed treatments (MANOVA, identity response, $F_{(8, 18)} = 2.82$, Wilks' Lambda, $p = 0.03$). Males followed (0.53 cm/s) trail speed treatment at a greater speed than (0.64 cm/s) and (1.23 cm/s) trail speed treatments (one way ANOVA, F ratio = 8.16, df = 2, $p < 0.01$). Also, males decreased the net distance they tracked a trail as the trail speed treatment increased, (one way ANOVA, F ratio = 7.52, df = 2, $p < 0.01$). In the fourth experiment (Table 4), there was not a significant difference in the mean values of kinematic metrics between trail speed treatments (MANOVA, identity response, $F_{(8, 4)} = 1.94$, Wilks' Lambda, $p = 0.27$).

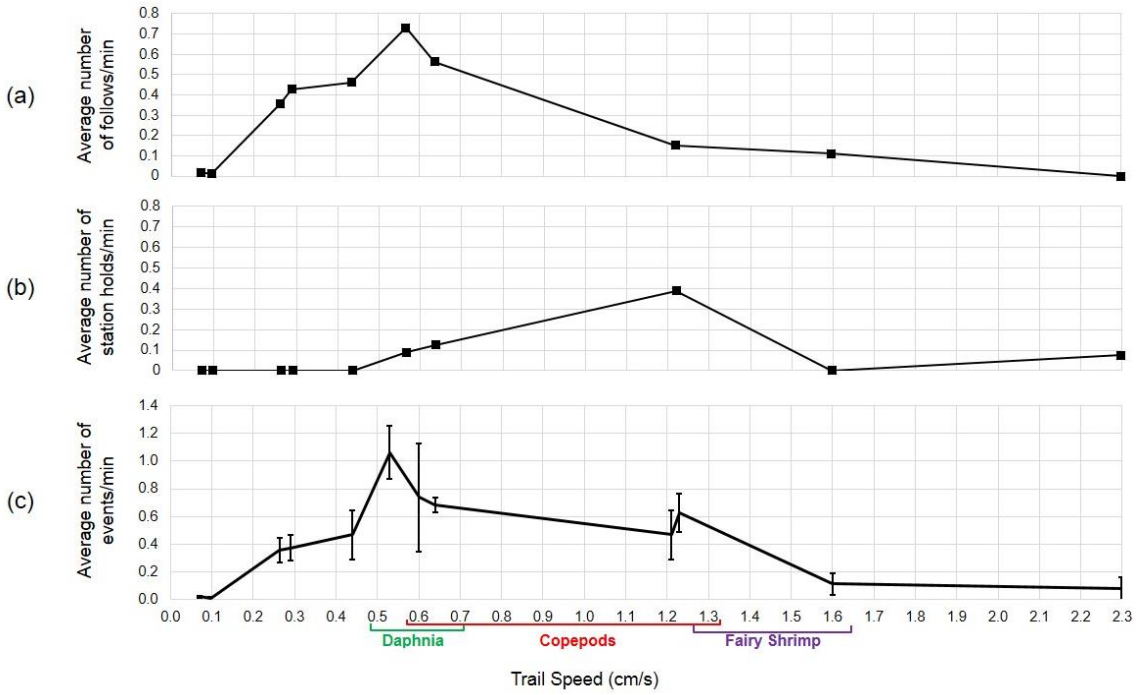

Figure 4: Males modify their trail-following behavior with a change in trail speed. (a) The mean number of following events for a given trail mimic speed treatment. A general trend shows that as the trail mimic speed increased, the mean number of following events decreased. (b) The mean number of station holding events for a given trail mimic speed treatment. The behavior of station holding events was only observed at trail speeds equal to and greater than 0.53 cm/s, but was absent at the trail speed of 1.60 cm/s. (c) The frequency of activity between trail speed treatments. A general trend suggests that males increased their frequency of activity as the trail speed increased, with a peak between 0.53-0.60 cm/s.

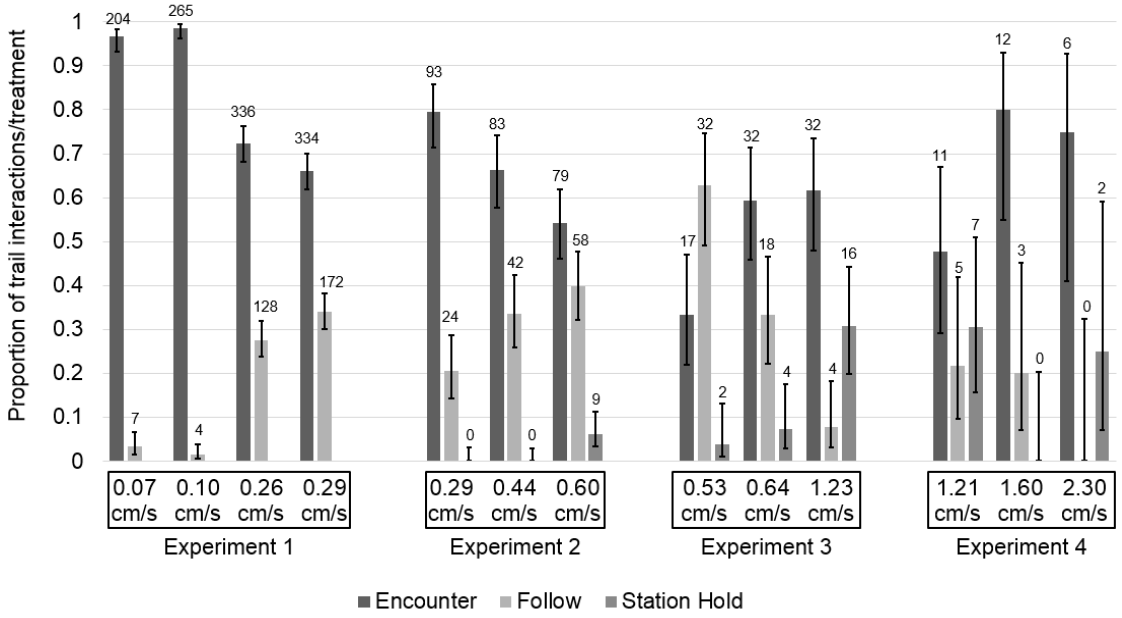

Figure 5: Proportion of copepod trail interactions classified as trail encounters, follows and station holding events between trail speed treatments with 95% confidence limits. In experiment 1, males followed the trail treatments 0.26 cm/s and 0.29 cm/s proportionally more than the trail treatments 0.07 cm/s and 0.10 cm/s. In experiment 2, males followed the trail speed treatment 0.60 cm/s proportionally more than the slower trail speed treatments, 0.29 cm/s and 0.44 cm/s. Males exhibited station holding behavior only on the fastest trail speed treatment (0.60 cm/s). In experiment 3, males followed the trail speed treatment of 0.53 cm/s proportionally more than the trail speed treatments of 0.64 cm/s and 1.23 cm/s. Males exhibited station holding behavior on all trail speed treatments, but this behavior was proportionally greater on the fastest trail speed (1.23 cm/s). In experiment 4 there were no observable trends or significant differences between the trail speed treatments in the proportion of encounters, follows, or station holding events.

Table 4: A comparison of kinematic metrics between trail speed treatments. In experiment 2, there was a significant difference in the mean values of kinematic metrics between trail speed treatments (MANOVA, identity response, $F_{(8, 12)} = 3.33$, Wilks' Lambda ($p = 0.03$). The speed at which males followed a trail was slower for the (0.60 cm/s) trail speed treatment compared to trail speeds of (0.29 cm/s) and (0.44 cm/s) (one-way ANOVA, F ratio = 17.36, df = 2, $p < 0.001$*). In experiment 3, there was a significant difference in the mean values of the kinematic metrics between the trail speed treatments (MANOVA, identity response, $F_{(8, 18)} = 2.82$, Wilks' Lambda, $p = 0.03$). Males followed (0.53 cm/s) trail speed treatment at a greater speed than (0.64 cm/s) and (1.23 cm/s) trail speed treatments (one way ANOVA, F ratio = 8.16, df = 2, $p < 0.01$*). Males decreased the net distance they tracked a trail as the trail speed treatment increased, (one way ANOVA, F ratio = 7.52, df = 2, $p < 0.01$*). The values of the kinematic metrics are expressed as the mean value ± standard deviation.

Experiment	Flow Speed (cm/s)	Duration of tracking event (sec)	Average Trail Tracking Speed (cm/s)	Net Distance Tracked (cm)	Gross Distance Tracked (cm)
1	0.07	1.30 ± 0.57	1.31 ± 0.39	1.29 ± 0.93	1.91 ± 1.29
1	0.10	1.42 ± 1.58	1.50 ± 0.31	1.36 ± 1.19	2.00 ± 2.06
1	0.26	1.51 ± 0.81	1.33 ± 0.21	2.19 ± 1.75	2.63 ± 1.90
1	0.29	1.48 ± 0.45	1.28 ± 0.17	2.00 ± 0.69	2.41 ± 0.79
2	0.29	1.17 ± 0.35	**1.02 ± 0.15***	1.34 ± 0.39	1.56 ± 0.48
2	0.44	2.07 ± 0.70	**0.76 ± 0.28**	1.86 ± 0.31	2.53 ± 0.87
2	0.60	2.32 ± 1.02	**0.26 ± 0.02**	1.44 ± 0.59	2.31 ± 1.29
3	0.53	2.78 ± 0.43	**0.82 ± 0.30***	**2.39 ± 0.69***	3.87 ± 0.65
3	0.64	3.10 ± 1.96	**0.42 ± 0.17**	**1.58 ± 1.00**	3.17 ± 1.89
3	1.23	2.34 ± 1.33	**0.30 ± 0.13**	**0.59 ± 0.37**	2.37 ± 1.58
4	1.21	5.31 ± 4.15	0.43 ± 0.28	1.46 ± 1.05	4.92 ± 3.90
4	1.60	1.92 ± 0.21	0.31 ± 0.36	1.36 ± 0.04	2.71 ± 0.42
4	2.30	1.73	1.47	0.79	1.82

1.4 Discussion

1.4.1 Remote mate detection: chemical or hydrodynamic cues?

Results from this study show that males of the freshwater copepod species *Hesperodiaptomus shoshone* (Wyoming) did not discriminate and followed trail mimics that were scent-less in the same way as trails that were scented with female and male diffusible chemical exudates. Using three-dimensional videography, males showed the same lack of discrimination for sex-specific chemical cues by following the wakes produced by conspecific females and males. During mate pursuit, male mate-tracking behavior did not differ between males tracking another male as compared to a female copepod. The lack of difference in tracking behavior in the mating and chemical trail experiments suggests that (a) males and females do not produce a diffusible chemical exudate and/or (b) the male copepod is unable to distinguish the odorant of the female from that of the male. In either case, chemical cues apparently do not mediate the mate-tracking behavior of male copepods in this freshwater species.

The importance of hydrodynamic cues in finding a mate was assessed by exposing males to scent-less trail mimics where the flow speeds of the trail mimic were matched to a range of lake resident swimming speeds. Although the experimental design does not permit statistical analysis across the trials with different flow speed ranges, the results strongly suggest that the male copepod showed a speed-related modification in its response to a continuous scale of flowing trail mimics. Intermediate speeds, similar to that of the swimming speed of *H. shoshone*, evoked the highest frequency of activity and, in general, trails were followed for a longer period of time and greater gross distance. Lower speeds, below that of the swimming speeds of lake residents, evoked a lower frequency of activity and, in general, trails were followed for a shorter period of time and shorter gross distance. Comparing chemical scented trails to hydrodynamic trails at the same range of trail speeds (0.24 cm/s to 0.29 cm/s), males did not differ in their frequency of activity (Figure 6). An increase was found in the frequency of activity when

males were exposed to trail speeds that mimicked a range of swimming speeds of similar magnitude to the swimming speeds of conspecific males and females.

These multiple lines of evidence confirm that males of *H. shoshone* (Wyoming) follow continuous hydrodynamic trails left by smoothly swimming copepods for remote mate detection. This is in contrast to previous studies which have shown that copepods sensing hydrodynamic cues are reacting to discrete wakes of pulsed flow left by hopping or escaping copepods, not continuous hydrodynamic trails (Strickler, 1998; Fields & Yen, 2002; Doall et al. 2002; Strickler & Balaszi, 2007). The mate-finding behavior of *H. shoshone* (Wyoming) contrasts with other copepods that follow continuous trails for mate detection (Table 5) because *H. shoshone* (Wyoming) are not responding to female diffusible chemical exudates within the trail. The shared behavior between chemical trail trackers and *H. shoshone* is that females swim smoothly through the water and generate a continuous trail that males detect and follow to the cue source (Table 5). A key contrast is that chemical trail tracking males can initially detect a female scented trail from a remote distance, up to 30 body lengths away from the female source (Doall et al., 1998; Weissburg et al., 1998; Tsuda & Miller, 1998). Both populations of *H. shoshone* (Wyoming and California) initially detect a conspecific copepod trail at a much shorter distance, between 2-3 body lengths away from the cue releasing copepod. This short initial detection distance is similar to copepods that detect hydrodynamic disturbances from hopping animals from an initial distance of 1-2 body lengths from the source (Doall et al., 2002). Populations of *H. shoshone* contrast in that male *H. shoshone* (California) follow a female trail for up to 15 body lengths (Yen et al., 2011), whereas male *H. shoshone* (Wyoming) follow a trail for up to 5 body lengths. These important distinctions are supported by the fact that a hydrodynamic cue persists in the surrounding fluid medium for a much shorter period of time than a chemical cue (Bradbury and Vehrencamp, 2011). Even though *H. shoshone* (Wyoming) follows a continuous trail, they are reacting to a hydrodynamic cue and not a chemical cue. Hence, *H. shoshone*

(Wyoming) initially detects and follows a potential mate for a much shorter distance than known chemical trail tracking copepods.

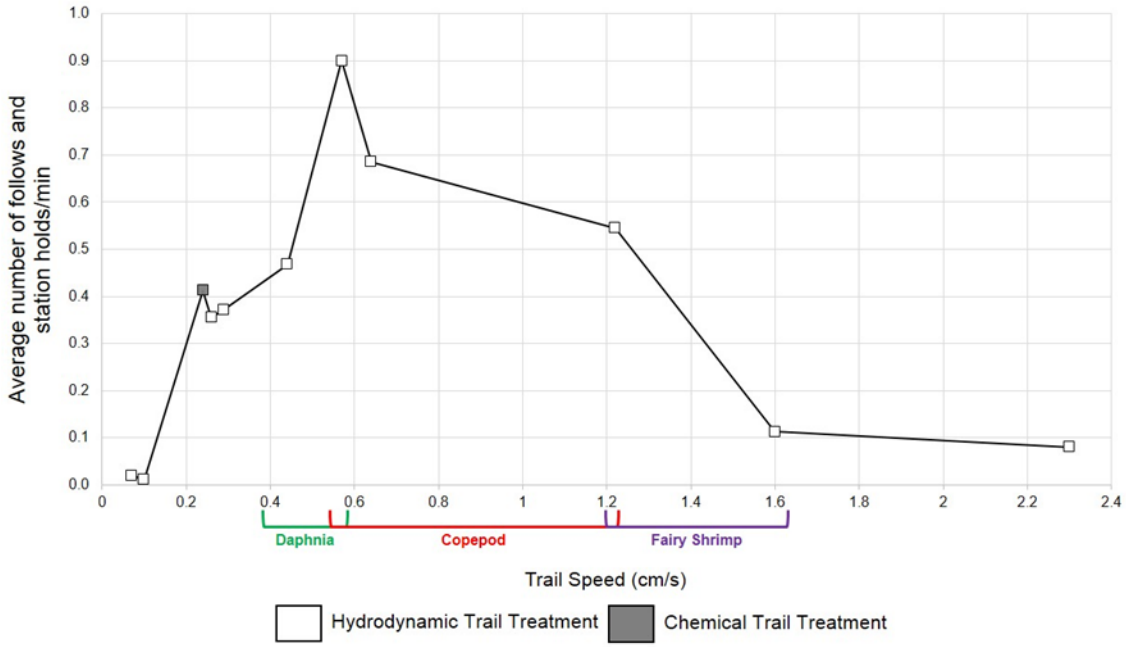

Figure 6: A comparison of the frequency of activity between chemical and hydrodynamic trail mimic experiments. Males did not differ in their frequency of activity for the chemical scented trails (grey box) compared to hydrodynamic trail treatments (white boxes) of similar speed (0.26 cm/s and 0.29 cm/s). The frequency of activity increased when males were exposed to trail speeds that mimicked a range of conspecific copepod speeds (0.57 cm/s, 0.64 cm/s, and 1.22 cm/s).

Table 5: A chart comparing key distinctions in the tracking behavior of copepods: *Hesperodiaptomus shoshone*, *Temora longicornis*, and *Centropages typicus*. The tracking behavior of *Hesperodiaptomus shoshone* (Wyoming) differs from that of other copepods for which chemical scented trail following behavior has been documented.

	***Hesperodiaptomus shoshone* (Wyoming)**	***Hesperodiaptomus shoshone* (California)**	***Temora longicornis***	***Centropages typicus***
Copepod length (mm)	2.43 ± 0.02	2.46 ± 0.13	1.1-1.2	1.5-1.7
Swimming Behavior	Cruising	Cruising	Cruising	Cruising
Initial detection distance (cm)	Short distance	Short distance	Long distance	Long distance
Pursuit distance (cm)	Short distance	Long distance	Long distance	Long distance
Time spent tracking (sec)	Short time	Long time	Long time	Long time
	Current study	Yen et al. (2011)	Doall et al. (1998)	Bagoien & Kiorboe (2005a)

1.4.2 Multiple cues for detecting and recognizing a mate

Hesperodiaptomus shoshone has exhibited behaviors that suggest they are likely to use contact chemical cues. Immediately after contact, a male would escape from another male but capture a female. This immediate reaction to surface contact is similar to the behavior observed in *Labidocera aestiva* (Blades, 1977). Males of the copepod species, *Labidocera aestivia*, grasp the caudal rami of a potential mate with their right antennule. If a male grasps a potential female mate at any other site than the caudal rami, then the male releases the female immediately (Blades, 1977). This behavior suggested that a male perceived a specific cue from the copepod caudal rami which indicated to either grasp or release the copepod (Blades and Youngbluth, 1979; Blades-Eckelbarger, 1991). There is an adaptive value of using contact chemical cues as another means for mate recognition. In crustaceans, the use of multiple cues has shown to minimize the number of mistakes made by males in mating with other males or sexually immature females (Lonsdale et al., 1998; Breithaupt and Thiel, 2008; Kamio et al., 2002). Since

mating is a critical process in maintaining species integrity, it should be protected by a "combination lock" (Hay, 2009), where a series of checkpoints mediated by chemical, hydrodynamic, and/or mechanical cues provide a "unique code" for successful mate recognition. The first checkpoint to mediate remote mate detection for many copepod species is the reliance on chemical scented trails (Colin, 1995; Doall et al., 1998; Tsuda & Miller, 1998; Bagoien & Kiorboe, 2005a; Kiorboe, Bagoien, Thygesen, 2005c; Goetze & Kiorboe, 2008; Goetze, 2008; Seuront, 2013; Yen et al., 2011). As a second checkpoint, some copepods rely on contact chemical cues to mediate mate recognition (Katona, 1973; Snell & Carmona, 1994; Lonsdale et al., 1996; Lonsdale et al., 1998; Frey et al., 1998; Ting et al., 2000). *Hesperodiaptomus shoshone* (Wyoming) may use multiple sensory detection methods and checkpoints for mate recognition. For remote mate detection, male *H. shoshone* may rely on continuous hydrodynamic trails. These trail-like cues may provide zooplankton specific information to the male receiver that the potential mate is a conspecific copepod. For further mate recognition, male *H. shoshone* may rely on contact chemical cues to discern the sex of the potential copepod mate.

1.4.3 Following continuous hydrodynamic trails: An adaptive behavior for life in fast flowing currents

Male *H. shoshone* exhibited a novel trail reaction for copepods, such that they would station hold within high velocity trails. Station holding has been documented in fish species that live in fast flowing currents, in which they swim into the flow and hold their position to avoid being swept downstream (Montgomery et al., 1997). It was proposed that animals may station hold to avoid lake exodus or else they may lose access to vital resources such as mates, food, and shelter (Carlson and Lauder, 2010). Perhaps a males' ability to follow hydrodynamic trails could be an evolutionary adaptive advantage to avoid lake exit. Animals that live in fast flowing currents are typically exposed to lake exit flow velocities in the range of 29-38 cm/second (Miller et al. 2006), which is

considerably higher than the maximum trail velocity of 2.30 cm/second that males of *H. shoshone* were exposed to in this study. However, *Hesperodiaptomus shoshone* live in high alpine lakes in which the exit flow velocity is likely to vary based on the season, the influx of water from draining or filling of connected water sources, and the outflow geometry of the lake. Therefore, they could be subjected to a wide range of exit flow velocities, including those they were exposed to in this study. The range of exit flow velocities that *H. shoshone* may be exposed to needs to be examined to assess the adaptive value of station holding in avoidance of lake exit.

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